DEVELOPMENTAL BIOLOGY 174, 345–359 (1996)
ARTICLE NO. 0079

Multiple Actions of Stem Cell Factor in Neural

Carol J. Langtimm-Sedlak,* Brett Schroeder,* Jeanette L. Saskowski,* Josette F. Carnahan,† and Maya Sieber-Blum*,[1]

Department of Cellular Biology and Anatomy, Medical College of Wisconsin, Milwaukee, Wisconsin 53226; and †Department of Neurobiology, Amgen Center, Thousand Oaks, California 91320

The neural crest is a transient tissue of the vertebrate embryo that gives rise to most primary sensory neurons and pigment cells in the adult organism, among other cell types and tissues. Many neural crest cells are pluripotent in the sense that their progeny can generate more than one phenotype. The presence of pluripotent neural crest cell-derived cells at sites of terminal differentiation suggests that location-specific cues from the embryonic environment, such as growth factors, are involved in directing their survival, proliferation, and cell type specification. We have therefore examined the influences of one pertinent growth factor, stem cell factor (SCF), on neural crest cell development by *in vitro* colony assay in a serum-free culture medium. SCF showed three major effects. (1) SCF is trophic for early neural crest cells, that is, either pluripotent cells and/or their more mature progeny. This effect occurs only if SCF is present throughout the culture period, and it is not observed when a neurotrophin is present in addition to SCF. (2) More colonies contain sensory neuron precursors in the presence of SCF. This effect is neutralized by NGF and neurotrophin-3 (NT-3), but not by brain-derived neurotrophic factor (BDNF). (3) The combination of SCF and any neurotrophin tested (NGF, BDNF, NT-3) is trophic for melanogenic cells, whereas SCF alone does not detectably affect melanogenesis. This suggests either that both types of factor are required for melanotrophic action or that melanogenic cells become dependent on neurotrophins after exposure to SCF. Our observation that SCF is required during the first half of the culture period only, and NGF during the second half only, indicates the latter possibility. Whereas coat color changes in the mouse mutants *W* (*c-kit* defect) and *Steel* (SCF defect) and several *in vivo* and *in vitro* studies by other investigators have shown previously that SCF is melanotrophic, they also indicated the requirement of an additional factor, or factors, in melanogenesis. Our data suggest that SCF affects neural crest cell development at multiple levels and that survival of melanogenic cells is mediated by a combination of SCF and a neurotrophin, rather than by SCF alone.

INTRODUCTION

Derivatives of the neural crest include most primary sensory neurons and the melanocytes of the skin and internal organs among many other structures (Weston, 1970; Noden, 1978; Le Douarin, 1982; Gershon *et al.*, 1993).

While some neural crest cells appear to be committed early to the melanogenic, sensory neuron, and smooth myogenic lineages (Sieber-Blum and Cohen, 1980; Sieber-Blum, 1989a; Ito and Sieber-Blum, 1991), many are pluripotent in the sense that they are able to generate progeny of two or more differentiated phenotypes (Sieber-Blum and Cohen, 1980; Sieber-Blum and Sieber, 1984, 1985; Baroffio *et al.*, 1988; Bronner-Fraser and Fraser, 1988; Sieber-Blum, 1989a; Fraser and Bronner-Fraser, 1991; Ito and Sieber-Blum, 1991; Ito *et al.*, 1993). Pluripotent neural crest cells are also present at sites of terminal differentiation, including the dorsal root ganglia and the developing skin and persist well into advanced embryonic development (Duff *et al.*, 1991; Richardson and Sieber-Blum, 1993; Ito and Sieber-Blum, 1993). This suggests that signals from the embryonic microenvironment play a role in the survival, proliferation, and differentiation of neural crest cells. Growing evidence in different experimental systems indicates that peptide growth factors provide some of these signals (Stemple *et al.*, 1988; Cattaneo and McKay, 1990; Birren and Anderson; 1990; Sieber-Blum, 1991; Weston, 1991; Zhang and Sieber-Blum, 1992; Kalcheim *et al.*, 1992; Morrison-Graham *et al.*, 1992; Vogel *et al.*, 1993; Sieber-Blum *et al.*, 1993; Zhang *et al.*, 1993). In

[1] To whom correspondence should be addressed.

0012-1606/96 $18.00

the present study, we have examined the effects on neural crest cell differentiation of one pertinent growth factor, stem cell factor (SCF), alone and in combination with neurotrophins.

SCF is also known as Steel factor, mast cell growth factor, or kit ligand (Anderson *et al.,* 1990; Copeland *et al.,* 1990; Huang *et al.,* 1990; Martin *et al.,* 1990; Williams *et al.,* 1990; Zsebo *et al.,* 1990a, b). SCF is defective in the mouse mutant *Steel* (*Sl*) (Mayer, 1970; Altus *et al.,* 1971; Fried *et al.,* 1973) and is required for the development of hematopoietic stem cells, primordial germ cells, and neural crest-derived melanocytes (reviewed by Silvers, 1979; Russell, 1979; Geissler *et al.,* 1981). Studies *in vitro* (Morrison-Graham *et al.,* 1990; Murphy *et al.,* 1992; Morrison-Graham and Weston, 1993; Lahav *et al.,* 1994) and *in vivo* (Steel *et al.,* 1992; Nishikawa *et al.,* 1991) have shown that SCF is required for the survival of neural crest-derived melanocyte precursors.

The receptor for SCF has been identified as the product of *c-kit,* a proto-oncogene tyrosine kinase that is homologous to the receptors for CSF-1 and PDGF (Chabot *et al.,* 1988; Geissler *et al.,* 1988; Qui *et al.,* 1988) and is defective in the mutant mouse *Dominant White Spotting* (*W*); Bennett, 1956; McCulloch *et al.,* 1964; Geissler *et al.,* 1981). Since prior *in vitro* studies were performed in complex culture media and because there are indications for a requirement of an additional factor in melanogenesis (Morrison-Graham *et al.,* 1990; Murphy *et al.,* 1992; LeCoin *et al.,* 1995) we reevaluated the role of SCF in a serum-free culture medium by *in vitro* colony assay.

There were three major observations. SCF is trophic for pluripotent neural crest cells and/or their immediate progeny. It enhances differentiation or survival of sensory neuron precursors, and it requires the presence of a neurotrophin for melanotrophic activity.

MATERIALS AND METHODS

Materials. MCDB 201, bovine serum albumin (BSA), gentamicin, prostaglandin E_1, tocopherol, progesterone, β-hydroxybutyrate, cobalt chloride, biotin, oleic acid, insulin, ascorbic acid, putrescine, apo-transferrin, α-melanocyte stimulating hormone (α-MSH), retinoic acid, dexamethasone, 2.5S NGF from mouse submaxillary gland, and human recombinant NGF were purchased from Sigma Chemical Co. (St. Louis, MO). Triiodothyronine was purchased from Aldrich Chemical (Milwaukee, WI). Bovine dermal collagen was obtained from Celtrix Pharmaceuticals (Santa Clara, CA) and plasma fibronectin was purified from horse serum by the method of Yamada (1982). Recombinant chicken SCF was a gift from Amgen (Thousand Oaks, CA), while human recombinant neurotrophin-3 (NT-3) and brain-derived neurotrophic factor (BDNF) were a gift from Genentech (South San Francisco, CA). Bromodeoxyuridine (BrdU) was purchased from GIBCO BRL (Grand Island, NY) and antibodies to BrdU were obtained from Becton–Dickinson (San Jose, CA). AC-4, a monoclonal antibody that recognizes stage-specific embryonic antigen-1 (SSEA-1), was a gift from Drs. J. Dodd and T. Jessell (Dodd and Jessell, 1986). Neuron-specific monoclonal antibody to β-III tubulin was received from Dr. A. Frankfurter (Lee *et al.,* 1990). Polyclonal antibodies against dopamine-β-hydroxylase (DBH) were purchased from Eugene Tech Int. (Ridgefield Park, NJ). MelEM antibody was obtained from the Hybridoma Bank (Iowa City, IA). Rhodamine- and fluorescein isothiocyanate (FITC)-conjugated secondary antibodies were purchased from Organon Teknika (Westchester, PA) and preabsorbed subtype-specific antibodies were obtained from Jackson Laboratories.

Neural crest cell primary cultures. Neural crest cell cultures were prepared as described (Sieber-Blum and Cohen, 1980; Sieber-Blum and Sieber, 1985). Briefly, the last six segments of Hamburger and Hamilton (1951) stage 14 quail embryos were excised using an electrolytically sharpened tungsten needle and trypsinized. The neural tubes were isolated from the surrounding tissues by trituration and placed into 35-mm culture dishes coated with a collagen gel. The cells were cultured in a serum-free medium that has been described previously (Sieber-Blum and Chokshi, 1985), with the modifications that MCDB 201 was substituted for MCDB 202 and all peptide growth factors were omitted in control cultures. The culture medium was supplemented with 0.5% Day 11 chick embryo extract and 1.25 μg/ml gentamicin. The cultures were incubated at 36.5°C in a humidified atmosphere containing 5% CO_2 and 10% O_2.

Colony assay. Colony assays were performed as described (Sieber-Blum and Cohen, 1980; Sieber-Blum and Sieber, 1985). Briefly, the neural tubes were removed 24 hr after explantation, leaving behind the neural crest cells which had migrated onto the plate. The neural crest cells were resuspended by trypsinization. The density of the essentially single-cell suspension was adjusted to 250 cells per milliliter. One-milliliter aliquots of this cell suspension were added to culture dishes coated with a collagen gel (Kleinman *et al.,* 1979) and conditioned for 1 hr with 1 ml of culture medium containing 50 μg of plasma fibronectin in the presence or absence of growth factor(s) as indicated. Viable cells firmly attached to the substratum within $\frac{1}{2}$ hr. Each experimental and control series consisted of 10–12 replicate dishes. The culture medium and growth factors were changed every day for the first 6 days of culture and then again on Culture Day 8. For those cultures processed for indirect immunofluorescence with antibodies against SSEA-1 and DBH, the number of unpigmented, pigmented, and mixed colonies was enumerated in each plate on Culture Day 8 with phase-contrast optics. On Culture Day 10, the plates were processed for indirect immunofluorescence as described and the number of colonies containing SSEA-1- and DBH-immunoreactive cells was determined with a Leitz Diaplan fluorescent microscope. For those cultures treated with BrdU and visualized using indirect immunofluorescence, the plates were maintained until Culture Day 3 or 6 and then processed as described. The number of BrdU-immunoreactive cells per colony was determined with a Leitz Diaplan fluorescent microscope. Statistical analysis for all experiments was performed using the two-sided Student's *t* test. A *P*-value of ≤ 0.05 indicated significant differences in experiments consisting of two groups. When multiple comparisons were made, significance levels were corrected according to Dunnett (1964): with three groups $P \leq 0.03$, with four groups $P \leq 0.02$, with six groups $P \leq 0.012$, and with eight groups $P \leq 0.009$ were considered indicative of significant differences.

Growth factors. Hundredfold stock solutions of recombinant chicken SCF were prepared in Hanks' Balanced Salt Solution (HBSS) and stored at 4°C. NGF was diluted with 0.1% BSA in calcium- and magnesium-free PBS (CMF-PBS) to prepare a stock solution and frozen at −80°C until needed. NT-3 and BDNF were diluted in 0.1% acetic acid to obtain hundredfold stock solutions, which

were stored at 4°C. SCF, NT-3, and BDNF were used at a final concentration of 10 ng/ml, while the final concentration of NGF was 20 ng/ml.

Indirect immunofluorescence. Cultures were stained as described previously (Sieber-Blum, 1989b) with monoclonal antibodies against SSEA-1, polyclonal antibodies against DBH, and monoclonal (clone TuJ1) or polyclonal antibodies against neuron-specific class III β-tubulin (Lee *et al.*, 1990; Katsetos *et al.*, 1994). Briefly, cultures were rinsed once with CMF-PBS and then fixed for 1 hr on ice with 4% paraformaldehyde in CMF-PBS. They were subsequently rinsed three times for 10 min each in CMF-PBS containing 0.1% BSA. The plates were then incubated overnight at 4°C with primary antibodies (pooled in the case of double labeling) in CMF-PBS with 0.1% BSA and 0.1% Triton X-100. The following day, the plates were again rinsed three times for 10 min each in CMF-PBS with 0.1% BSA. Secondary antibodies (rhodamine- and fluorescein-conjugated goat anti-mouse IgG, goat anti-rabbit IgG; preabsorbed goat anti-mouse IgG1-specific and preabsorbed goat anti-mouse IgM-specific antibodies) were added at 1:20 and the plates incubated for 1 hr in the dark at room temperature. In the case of double labeling, secondary antibodies were pooled before addition to the plates. After rinsing again in CMF-PBS containing BSA, 1 μg/ml Hoechst stain 33258 (bisbenzimide; Riedel-DeHaeng) was added to the plates for 15 min. The plates were then rinsed twice with CMF-PBS and mounted with 50% glycerol [containing 1 mg/ml phenylenediamine (PPD) in PBS, pH 8.5] and a glass coverslip.

For those cultures stained with antibodies to BrdU, the protocol was as follows. The cultures were incubated for 30 min at 36.5°C with 10 μM BrdU in culture medium. The plates were rinsed with HBSS and incubated on ice for 20 min in 0.5% paraformaldehyde. They were subsequently rinsed with CMF-PBS with 0.1% BSA, incubated containing 2 *N* HCl with 0.5% Tween 20 for 8 min, and washed with sodium borohydrate and subsequently with CMF-PBS-BSA before adding the primary antibodies. The anti-BrdU antibodies were diluted in 0.5% Tween 20 in PBS and the incubation time was 2 hr at room temperature. Fluorescein-conjugated secondary antibodies were used at 1:10 dilution. Fluorescence was observed either with a Leitz Diaplan fluorescence microscope or with a confocal microscope.

Confocal microscopy. Digital fluorescence images were viewed and captured using a Bio-Rad MRC-600 Laser Scanning Confocal Imaging System. The secondary antibodies of rhodamine and fluorescein were excited by a krypton/argon laser using the 568-nm and 488-nm laser lines, respectively, and captured as 8-bit grayscale images at a magnification of 40X using a Nikon 40 Fluor/1.30 oil objective mounted on a Nikon Optiphot microscope. The captured images were converted to a Tag Image File Format (TIFF) bitmap, and a pseudocolor look-up table was added to the TIFF images. The TIFF images were set up as slides using Microsoft PowerPoint and photographs were taken using a Focus Graphics Image Corder Plus film recorder.

DOPA reaction. The DOPA reaction was performed as described (Ito *et al.*, 1993).

RESULTS

Effects of SCF. In the presence of SCF (10 ng/ml), there were two consistent and significant changes. (1) The number of cells per colony in unpigmented and mixed colonies and (2) the number of colonies per plate containing sensory neuron precursors that express the SSEA-1 epitope were increased (Figs. 1 and 2; Table 1). SSEA-1 has been determined to be a marker specific for cells in the sensory neuron lineage in quail neural crest derivatives (Sieber-Blum, 1989b; Rachel and Sieber-Blum, 1989). By contrast, the total number of colonies per plate, the number of colonies containing DBH-positive cells (Table 1; Fig. 1), and the number of SSEA-1-immunoreactive cells per SSEA-1-positive colony (data not shown) were unchanged. Using colony size as a measure, it was determined that SCF concentrations from 10 to 20 ng/ml gave a maximal response (Fig. 3). All subsequent experiments were performed with 10 ng/ml of SCF.

Contrary to expectation, neither the number of pigmented colonies per plate nor the number of cells per pigmented colony was detectably affected. Within mixed colonies, there was a preferential increase in the number of unpigmented cells, while the number of melanocytes per colony was the same as in the control, even after the DOPA reaction was performed (Table 2). These data are in disagreement with those of Lahav *et al.* (1994), who reported an increase in presumptive pigment cells (unpigmented melanoblasts) and in melanocytes upon treatment with SCF. We therefore repeated the above experiments using, like Lahav and collaborators, the early melanocyte marker MelEM, rather than pigmentation, as a criterion for melanogenesis. MelEM is thought to bind not only to pigmented cells, but also to committed melanocyte precursors before they become pigmented (Nataf *et al.*, 1993). Colony assays were performed in the presence and absence of SCF and the cells were triple stained with polyclonal antibodies against the neuron-specific β-III tubulin (Lee *et al.*, 1990), the monoclonal MelEM ab, and bisbenzimide for total nuclear counts. At Colony Culture Day 2, all MelEM-immunoreactive cells were unpigmented and also bound the neuron-specific β-III tubulin antibody (Figs. 4A and 4B). The β-III tubulin staining pattern in MelEM$^+$ cells was characteristically filamentous, but intensity of fluorescence was lower than in β-III tubulin$^+$/MelEM$^-$ cells. Intensely immunoreactive β-III tubulin$^+$/MelEM$^-$ cells had either a flattened (Fig. 4A) or a rounded and neuronal (Fig. 4C) morphology. The phenotypes defined by the two antibodies, β-III tubulin$^+$/MelEM$^-$ cells, and β-III tubulin$^+$/MelEM$^+$ cells were enumerated and correlated with total nuclear counts per colony. At Day 3, no statistically significant differences were observed between SCF and control cultures. By Day 4, MelEM-immunoreactive cells were pigmented, and most of them were β-III tubulin-negative. Rare lightly pigmented Mel$^+$ cells were observed that still showed traces of β-III tubulin immunoreactivity. A statistically significant increase in the portion of β-III tubulin-positive cells, but not of MelEM-positive cells, was observed in the presence of SCF at Day 4 (Table 3). These data indicated to us that we did not exclude unpigmented committed melanogenic cells by using pigmentation as a marker for melanocytes. They also suggested that the MelEM epitope is not unique to committed melanogenic cells.

Effects of SCF plus NGF. Since in our colony assay

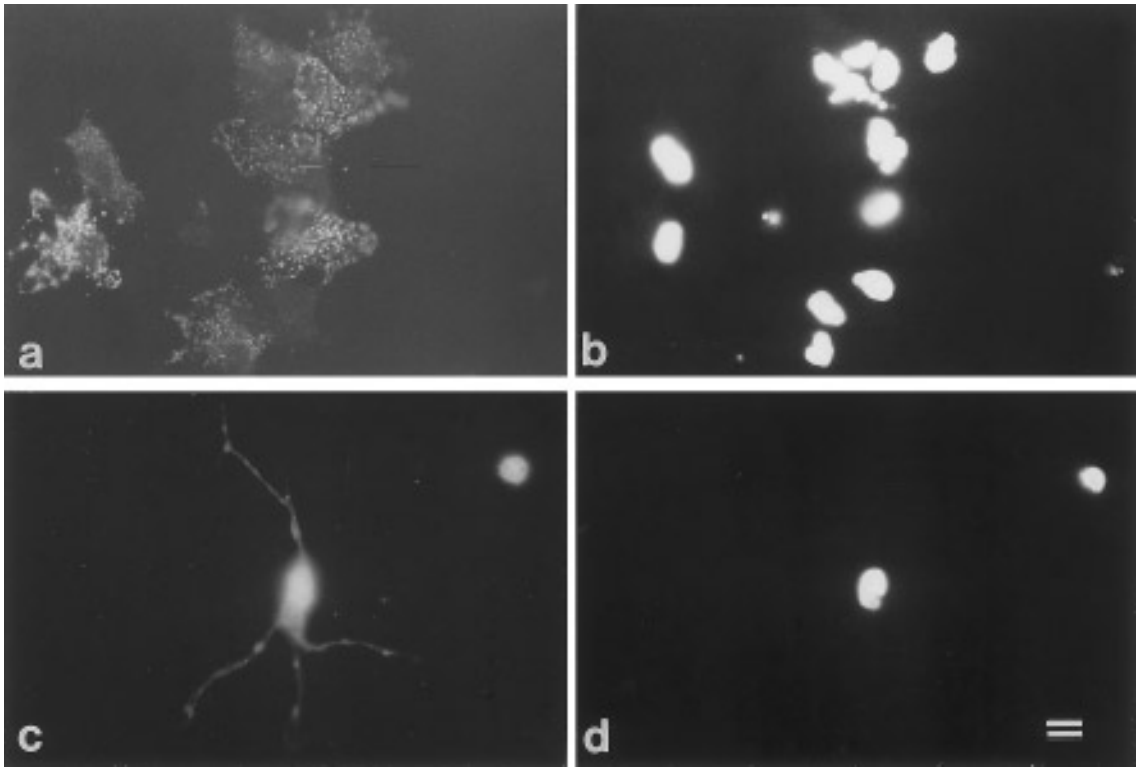
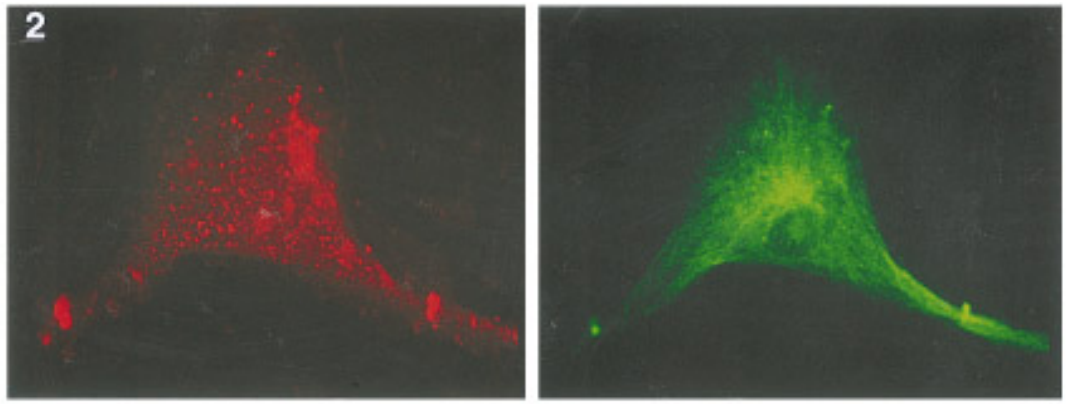


FIG. 1. Immunohistochemical staining of neural crest cells, Day 10 of culture. (a) SSEA-1-positive cells in an unpigmented colony and (b) Hoechst stain of the same field. (c) A DBH-positive cell in a mixed colony. Note the multipolar processes and varicosities typical of adrenergic neuroblasts. (d) The corresponding Hoechst stain for the field shown in (c). Bar, 10 μm.

pigment cell formation was not affected by SCF and because melanocytes can express NGF receptors, we examined whether a combination of SCF plus NGF affects melanogenesis. In the presence of both factors, SCF plus NGF, the number of cells per colony in pigmented colonies was indeed significantly increased, whereas NGF alone had no effect (Table 4). There was no longer an increase in the size of unpigmented and mixed colonies or in the number of SSEA-1-positive colonies per plate when the cells were cultured in the presence of the SCF/NGF combination (Table 4).

In order to exclude that the NGF-related effect was due to a contaminant from the mouse submaxillary gland extract, human recombinant NGF was used (Table 5). The increase in the size of pigmented colonies was also observed with human recombinant NGF in combination with SCF. The number of colonies per plate was not affected by either SCF or NGF (Tables 1, 2, and 4), suggesting that the factors have no effect on plating efficiency. To further exclude the possibility that selective cell attachment was responsible for the observed SCF/NGF effect on melanogenesis, the two factors were first added 4 hr after plating, that is, $3\frac{1}{2}$ hours after cell

FIG. 2. SSEA-1/β-tubulin double label. Since in serum-free medium, many SSEA-1-positive cells do not have the morphology of mature sensory neurons, they were double labeled with antibodies against the neuron-specific β-III tubulin. On the left, an SSEA-1-positive cell was imaged by confocal microscopy (projected image; rhodamine fluorescence). On the right one intracellular plane of the same cell shows filamentous β-III tubulin immunoreactivity (fluorescein fluorescence). Many, but not all, SSEA-1-positive cells were also β-III tubulin-positive. This discrepancy is to be expected because SSEA-1 is already expressed by mitotic cells (Scott, 1993), whereas β-III tubulin immunoreactivity is first observed in postmitotic cells (A. Frankfurter, personal communication). Magnification, approximately 384-fold.
FIG. 4. Colocalization of MelEM with neuron-specific β-III tubulin. Triple stain at Colony Culture Day 2 and Day 3 with bisbenzimide (Hoechst nuclear stain; A1, B1, C1), antibodies against β-III tubulin (rhodamine; A3, B3, C2, C3), and MelEM monoclonal antibody (A2, B2, C4). (A1–3) Culture Day 2; three of four visible cells are MelEM-positive and β-III tubulin-positive. One cell is intensely β-III tubulin-immunoreactive, but MelEM-negative (arrows). (B1–3) Culture Day 3; a MelEM-positive cell is also faintly β-III-immunoreactive (exposure time in B3 is double of that in A3). (C1–4) β-III tubulin-immunoreactive neuronal cells at Day 3 are MelEM-negative; (C2) focus on upper left cell, (C3) focus on process of lower right cell. Bars, 10 μm. A, B, same magnification; C1–C4, same magnification.


2

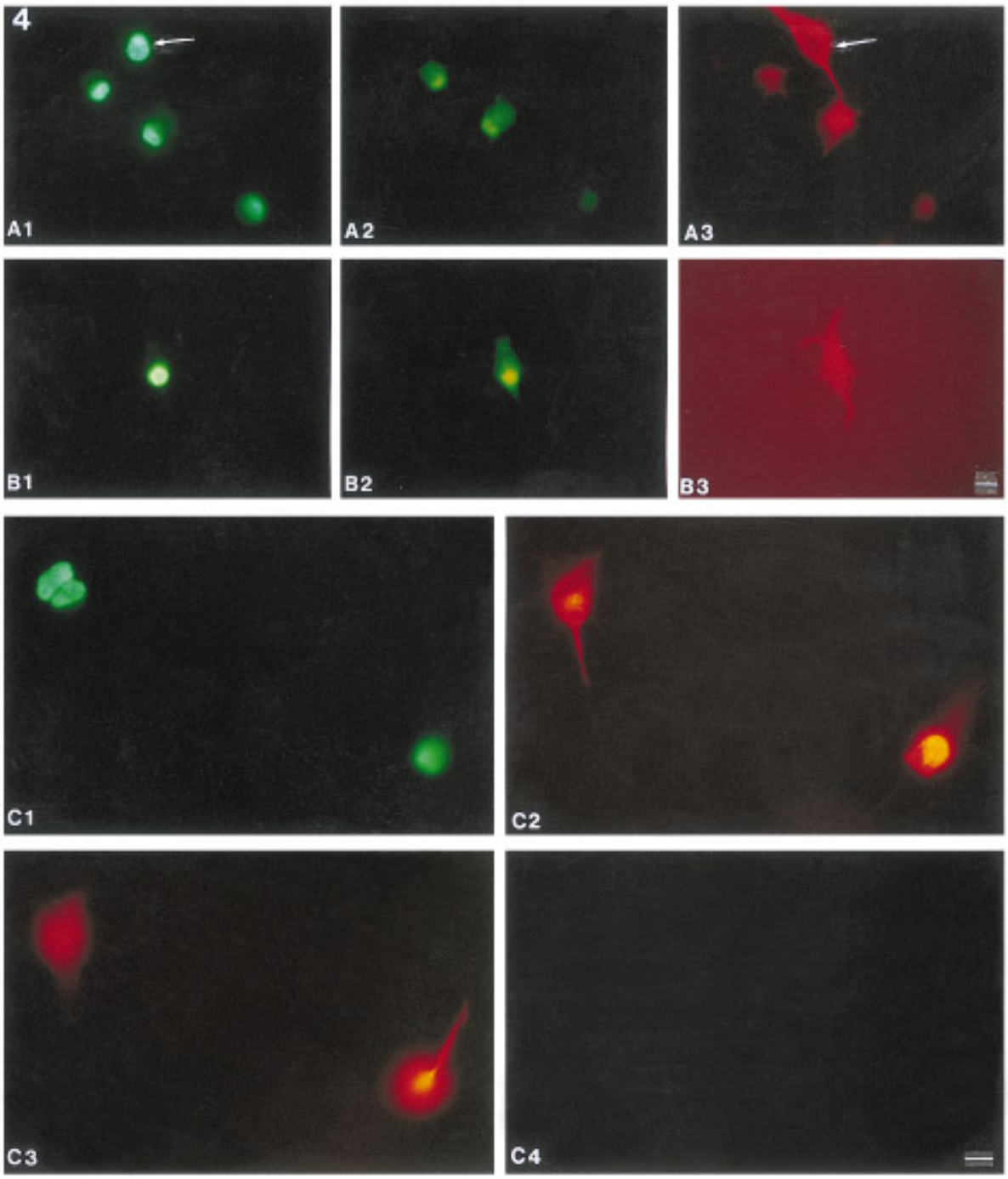

4
A1
A2
A3
B1
B2
B3
C1
C2
C3
C4

TABLE 1
Influence of SCF on Neural Crest Cell Development Assessed by *In Vitro* Colony Assay

Type of colony	Experimental condition	
	Control	SCF
	Number of colonies per plate ± SEM	
SSEA-1+	7.0 ± 0.7	10.9 ± 0.7 ($p = 0.001$)
DBH+	0.5 ± 0.2	0.7 ± 0.3 ($p = 0.65$)
Unpigmented	12.7 ± 1.2	10.3 ± 0.7 ($p = 0.11$)
Mixed	9.4 ± 1.0	7.8 ± 0.6 ($p = 0.19$)
Pigmented	23.3 ± 1.7	22.3 ± 1.9 ($p = 0.72$)
Total	45.6 ± 3.0	40.3 ± 2.2 ($p = 0.17$)
	Number of cells per colony ± SEM	
Unpigmented	12.1 ± 1.2	23.8 ± 2.8 ($p < 0.001$)
Mixed	15.1 ± 2.1	30.3 ± 3.2 ($p = 0.001$)
Pigmented	4.5 ± 0.3	5.1 ± 0.5 ($p = 0.30$)

Note. Quail neural crest cells were grown at clonal density in the presence or absence of 10 ng/ml chicken SCF. After 10 days, the cultures were triple labeled with (1) antibodies against SSEA-1 to identify cells in the sensory neuron lineage, (2) antibodies against DBH to identify sympathoadrenal cells, and (3) Hoechst nuclear stain for total nuclear counts. The number of colonies per plate containing SSEA-1-immunoreactive and/or DBH-immunoreactive cells was scored and categorized according to colony type. The total number of cells per colony was scored as well. Data are expressed as mean colony counts from 12 plates each. Cell counts are mean counts from 15–20 colonies for each colony type and experimental group. Statistical analysis is based on the two-tailed Student's *t* test. In another experiment, the increase in the number of SSEA-1^+ colonies per plate was from 12.7 ± 0.6 in the control to 14.5 ± 0.5 in the presence of SCF ($p = 0.02$) and the number of cells per colony increased from 9.8 ± 3.1 in the control to 18.2 ± 5.8 in the presence of SCF ($p = 0.002$) in unpigmented colonies, and from 29.1 ± 4.7 to 51.9 ± 5.0 in mixed colonies ($p = 0.004$).

attachment is completed. Delayed addition of growth factors did not change the results either (Table 5).

In order to determine whether the presence of both growth factors was required concurrently, they were added sequentially. The increase in size of unpigmented and mixed colonies and the increase in the number of SSEA-1 expressing colonies occurred only when SCF was present throughout the entire culture period. In contrast, the increase in the size of pigmented colonies also occurred when

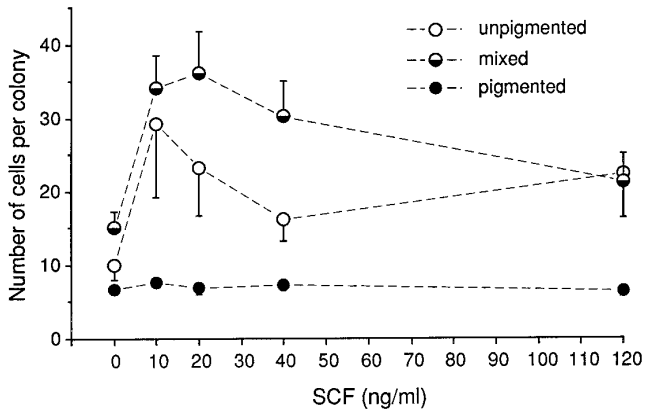


FIG. 3. Dose response of SCF. Colony assays were performed in the presence of various concentrations of SCF. The number of cells per colony were scored in unpigmented, mixed, and pigmented colonies. The optimal range of concentrations for trophic action on pluripotent cells was 10–20 ng/ml. No effect on pigment cells was observed, even at high concentrations of the factor.

SCF was present during the first half only and NGF during the second half only, but not vice versa (Table 6).

Trophic versus mitogenic effect. The observed increases in colony size could be due to either a mitogenic or trophic signal. To distinguish between the two possibilities, cells were exposed to BrdU at Day 3 or Day 6 of culture

TABLE 2
Analysis of Melanogenesis in the Presence of SCF after DOPA Reaction

Type of colony	Experimental condition		
	Control	SCF	SCF + DOPA
	Number of colonies per plate ± SEM		
Unpigmented	3.2 ± 0.6	3.8 ± 0.5 ($p = 0.48$)	3.0 ± 0.6 ($p = 0.82$)
Mixed	9.0 ± 1.2	11.0 ± 1.3 ($p = 0.26$)	10.6 ± 1.3 ($p = 0.36$)
Pigmented	130.5 ± 9.3	131.0 ± 8.8 ($p = 0.97$)	121.4 ± 16.3 ($p = 0.63$)
Total	142.7 ± 10.4	145.8 ± 9.0 ($p = 0.83$)	145.0 ± 10.8 ($p = 0.88$)
	Number of pigmented cells per mixed colony ± SEM		
Mixed	16.5 ± 3.2	16.6 ± 3.5 ($p = 0.99$)	16.0 ± 2.6 ($p = 0.90$)

Note. Cultures were reacted with DOPA, enhancing pigmentation in melanocytes, in order to exclude the possibility of omitting unpigmented or poorly pigmented melanocytes in the cell counts.

TABLE 3
Colony Assay Analyzed with Antibodies Against Neuron-Specific β-III Tubulin, MelEM, and Bisbenzimide at Colony Culture Day 4

Type of cells	Experimental condition	
	Control	SCF
Number of cells per mixed colony ± SEM		
Total number	14.2 ± 0.86	16.4 ± 0.51 ($p = 0.06$)
β-III Tubulin$^+$/Mel-EM$^-$ (neuronal)	2.0 ± 0.63	5.4 ± 1.16 ($p = 0.03$)
MelEM$^+$ (pigmented)	3.6 ± 0.67	3.0 ± 0.83 ($p = 0.59$)

Note. β-III tubulin-immunoreactive cells were intensely fluorescent and MelEM negative. All MelEM-positive cells were pigmented; faintly pigmented MelEM-positive cells showed traces of β-III tubulin immunofluorescence, whereas strongly pigmented cells were β-III tubulin-negative. The data were statistically analyzed with the Student's *t* test; $n = 5$ for each experimental group.

and subsequently processed for indirect immunofluorescence with anti-BrdU antibodies to visualize cells in S-phase. There were no significant differences in the portion of labeled nuclei between control and experimental groups (Table 7), indicating that the increase in the size of unpigmented and mixed colonies in the presence of SCF and of pigmented colonies in the presence of SCF plus NGF was due to a trophic, rather than a mitogenic signal. By contrast, parallel experiments with basic fibroblast growth factor and neurotrophins yielded significant differences (Zhang and Sieber-Blum, in preparation), corroborating the present data.

Neurotrophin specificity. To determine whether the observed effect on melanogenesis is specific for NGF or shared by other neurotrophins, NGF was replaced with BDNF or NT-3 (Table 8). Both neurotrophins mimicked the NGF effect, leading to increased pigmented colony size when combined with SCF (Table 8). Moreover, it was noted that the promoting effect of SCF on sensory neuron precursors was neutralized by NGF and NT-3, but not BDNF. BDNF, which by itself is involved in promoting differentiation of neural crest cells into sensory neuron precursors (Sieber-Blum, 1991), neither blocked nor increased the SCF effect. In addition, in the presence of one of the three neurotrophins tested

TABLE 4
Effect of SCF plus NGF

	Experimental condition			
	Control	SCF	NGF	SCF + NGF
Number of colonies/plate				
SSEA-1$^+$	4.3 ± 0.8	8.9 ± 0.8 ($p < 0.001$)	5.4 ± 0.5 ($p + 0.23$)	3.5 ± 0.7 ($p = 0.44$)
Unpigmented	8.9 ± 1.3	10.2 ± 0.7 ($p = 0.38$)	10.9 ± 0.8 ($p = 0.18$)	8.9 ± 1.1 ($p = 1.0$)
Mixed	10.8 ± 0.8	9.9 ± 1.0 ($p = 0.48$)	12.1 ± 1.7 ($p = 0.50$)	11.1 ± 0.9 ($p = 0.81$)
Pigmented	40.4 ± 2.2	46.7 ± 2.6 ($p = 0.08$)	44.0 ± 2.0 ($p = 0.24$)	42.8 ± 2.4 ($p = 0.47$)
Total	58.9 ± 3.6	66.8 ± 3.4 ($p = 0.13$)	67.0 ± 3.4 ($p = 0.12$)	62.6 ± 3.4 ($p = 0.47$)
Number of cells/colony				
Unpigmented	12.1 ± 2.2	22.9 ± 2.3 ($p = 0.002$)	12.9 ± 1.2 ($p = 0.75$)	19.3 ± 4.6 ($p = 0.16$)
Mixed	16.4 ± 3.1	26.8 ± 3.8 ($p = 0.04$)	18.9 ± 3.7 ($p = 0.61$)	12.6 ± 1.6 ($p = 0.29$)
Pigmented	4.7 ± 0.6	3.7 ± 0.4 ($p = 0.21$)	5.3 ± 0.8 ($p = 0.52$)	10.7 ± 1.0 ($p < 0.001$)

Note. Data are expressed as mean colony counts of 10 replicate dishes for each group and as mean cell counts of 20 colonies for each colony type. Statistical analysis is based on the two-tailed Student's *t* test. In order to correct for multiple comparisons, *p*-values of ≤ 0.02 are considered as indicative of significant differences.

TABLE 5
Control Experiment with Human Recombinant NGF and Late Addition of Growth Factors

Type of colony	Experimental condition			
	Control	SCF	NGF	SCF + NGF
	Number of colonies per plate ± SEM			
SSEA-1+	4.0 ± 0.3	7.9 ± 0.6 ($p < 0.001$)	4.3 ± 0.4 ($p = 0.58$)	2.6 ± 0.5 ($p = 0.04$)
Unpigmented	11.6 ± 1.5	11.0 ± 1.5 ($p = 0.78$)	12.8 ± 1.8 ($p = 0.62$)	11.8 ± 1.3 ($p = 0.92$)
Mixed	48.9 ± 3.1	47.1 ± 3.7 ($p = 0.71$)	48.7 ± 2.5 ($p = 0.96$)	46.1 ± 4.1 ($p = 0.59$)
Pigmented	116.1 ± 8.2	118.3 ± 8.7 ($p = 0.86$)	116.1 ± 8.2 ($p = 1.00$)	120.9 ± 7.9 ($p = 0.68$)
Total	176.6 ± 9.2	179.4 ± 6.5 ($p = 0.81$)	177.6 ± 7.5 ($p = 0.93$)	178.6 ± 10.0 ($p = 0.88$)
	Number of cells per colony ± SEM			
Unpigmented	11.6 ± 1.1	22.8 ± 5.2 ($p = 0.006$)	11.6 ± 1.4 ($p = 0.99$)	11.4 ± 1.2 ($p = 0.91$)
Mixed	20.3 ± 1.6	51.4 ± 5.1 ($p < 0.001$)	20.4 ± 1.7 ($p + 0.95$)	22.7 ± 1.9 ($p = 0.34$)
Pigmented	10.1 ± 0.9	10.0 ± 0.9 ($p = 0.94$)	10.3 ± 0.9 ($p = 0.87$)	29.8 ± 2.7 ($p < 0.001$)

Note. The same type of experiment as in Table 3, with the exceptions that (a) human recombinant NGF was used rather than mouse salivary gland-derived NGF and (b) the growth factors were added 4 hr after plating rather than before plating of the cells. The data are unchanged, indicating that (a) the melanogenic effect is due to NGF, not a contaminant and (b) prior addition of growth factors does not select for adhesion of subsets of neural crest cells. In order to control for multiple comparisons, p-values of $\leqslant 0.02$ are considered indicative of significant differences.

(NGF, BDNF, NT-3) the SCF-mediated increase in unpigmented and mixed colony size was neutralized (Table 8).

The data are summarized in Table 9.

DISCUSSION

SCF has three major effects on quail neural crest cell development as judged by *in vitro* colony assay. (1) SCF is trophic for pluripotent neural crest cells and/or their immediate progeny, if the factor is present throughout the culture period. This SCF-mediated effect was not observed when a neurotrophin was present as well. (2) More colonies contain sensory neuron precursors in the presence of SCF. This effect is neutralized by NGF and NT-3, but not by BDNF. (3) The combination of SCF and any neurotrophin tested (NGF, BDNF, NT-3) is trophic for melanogenic cells. SCF is required during the first half of the culture period only, NGF during the second half only. SCF alone has no detectable effect on melanogenesis in this colony assay.

Embryonic distribution of SCF, c-kit, neurotrophins, and neurotrophin receptors: Physiological relevance. Our data are consistent with the spatial and temporal expression in embryonic neural crest migratory pathways of SCF, the SCF receptor *c-kit*, neurotrophins, and neurotrophin receptors. In the avian embryo, SCF message is detected in the epidermis and *c-kit* message in the subectodermal mesenchyme that contains the presumptive melanocytes as early as on Embryonic Day (E) 4 (LeCoin *et al.*, 1995). In addition, there is evidence for an autocrine mechanism, in which neural crest cells themselves can synthesize and release SCF (G. Ciment, personal communication). In the mouse, migration of presumptive melanoblasts begins on E8.5 and the precursors are dispersed throughout the trunk ectoderm by E11.5 and into the limb bud by E12.5. Messenger RNA for SCF is detected by *in situ* hybridization in the mouse embryo as early as E9.5 (comparable to the 48-hr chick embryo) within the ventromedial and ventrolateral pathways of neural crest migration (Matsui *et al.*, 1990). By E12.5–13 presumptive melanoblasts have arrived in the skin of the developing limb bud and express *c-kit* transcripts (Orr-Urtreger *et al.*, 1990; Keshet *et al.*, 1991). Just prior to melanoblast colonization, cells in the dermis begin to express SCF transcripts (Keshet *et al.*, 1991).

Transcripts for SCF and *c-kit* are also detectable in the DRG during embryonic development of the mouse. They are first observed at Embryonic Day 12.5 and continue to be present throughout the remainder of embryonic develop-

TABLE 6
Sequential Addition of Growth Factors

Type of colony	Experimental condition					
	Control	SCF d0-d10	NGF d0-d10	SCF + NGF d0-d10	SCF d0-d5 NGF d6-d10	NGF d0-d5 SCF d6-d10
	Number of colonies per plate ± SEM					
SSEA-1$^+$	2.6 ± 0.4	6.2 ± 0.3 ($p < 0.001$)	2.7 ± 0.4 ($p = 0.87$)	2.8 ± 0.4 ($p = 0.75$)	2.5 ± 0.3 ($p = 0.86$)	2.9 ± 0.4 ($p = 0.59$)
Unpigmented	9.8 ± 0.1	8.4 ± 1.2 ($p = 0.36$)	9.0 ± 0.6 ($p = 0.50$)	7.5 ± 1.0 ($p = 0.11$)	9.0 ± 0.9 ($p = 0.55$)	9.1 ± 1.2 ($p = 0.66$)
Mixed	3.9 ± 0.5	6.1 ± 0.8 ($p = 0.03$)	4.6 ± 0.4 ($p = 0.32$)	4.7 ± 0.9 ($p = 0.46$)	3.4 ± 0.8 ($p = 0.61$)	4.7 ± 0.6 ($p = 0.31$)
Pigmented	7.1 ± 0.6	8.9 ± 0.7 ($p = 0.08$)	9.5 ± 1.1 ($p = 0.07$)	9.0 ± 1.4 ($p = 0.22$)	8.9 ± 0.7 ($p = 0.08$)	10.2 ± 1.2 ($p = 0.04$)
Total	20.8 ± 1.7	22.3 ± 1.4 ($p = 0.51$)	23.1 ± 1.4 ($p = 0.32$)	21.6 ± 2.2 ($p = 0.78$)	21.3 ± 0.9 ($p = 0.80$)	24.0 ± 1.9 ($p = 0.23$)
	Number of cells per colony ± SEM					
Unpigmented	25.9 ± 3.3	63.3 ± 8.3 ($p < 0.001$)	25.5 ± 3.7 ($p = 0.94$)	19.2 ± 2.5 ($p = 0.11$)	23.7 ± 2.8 ($p = 0.62$)	24.3 ± 3.3 ($p = 0.74$)
Mixed	31.6 ± 3.8	72.5 ± 8.5 ($p < 0.001$)	29.5 ± 4.6 ($p = 0.73$)	35.4 ± 4.9 ($p = 0.54$)	38.1 ± 4.0 ($p = 0.24$)	38.7 ± 6.7 ($p = 0.36$)
Pigmented	4.4 ± 0.6	3.7 ± 0.4 ($p = 0.29$)	3.9 ± 0.4 ($p = 0.45$)	11.7 ± 1.5 ($p < 0.001$)	10.7 ± 1.0 ($p < 0.001$)	4.3 ± 0.5 ($p = 0.90$)

Note. Control and experimental groups consisted of 10 replicate plates each and colony counts are mean values from 20 colonies for each colony type in each of the six groups. Statistical analysis is based on two-tailed Student's *t* test. In order to control for multiple comparisons, *p*-values of $\leqslant 0.012$ are considered indicative of significant differences.

ment (Orr-Urtreger *et al.*, 1990; Keshet *et al.*, 1991; Motro *et al.*, 1991).

NGF is a member of the family of neurotrophins, which also includes BDNF (Barde *et al.*, 1982), NT-3 (Hohn *et al.*, 1990; Rosenthal *et al.*, 1990; Maisonpierre *et al.*, 1990; Ernfors *et al.*, 1990), and neurotrophin-4/5 (NT-4/5, Berkemeier *et al.*, 1991). NGF supports the survival of neural crest-derived sympathetic and some sensory neurons both *in vivo* and *in vitro* (reviewed by Levi-Montalcini, 1982),whereas BDNF is trophic for sensory neurons. In the chick, cells in the dermal mesenchyme demonstrate low levels of transcripts for the low-affinity NGF receptor p75 from E5–10 (comparable to E14.5–17.5 of the mouse) and high levels in presumptive melanocytes of the feather follicles by E10 (Heuer *et al.*, 1990). Cultured human melanocytes express p75 mRNA and protein (Peacocke *et al.*, 1988; Yaar *et al.*, 1991). Yaar *et al.* (1994) have also shown that cultured human melanocytes express low levels of TrkA transcripts and that TrkA is quickly phosphorylated in the presence of NGF. *In situ* hybridization studies of the mouse embryo have revealed that NGF, BDNF, and NT-3 transcripts are expressed in the dermal mesenchyme from E11.5 to E17.5 (comparable to HH stages 12–36 in the quail embryo; Davies *et al.*, 1987; Schecterson and Bothwell, 1992). In addition, Yaar *et al.* (1991) and Di Marco *et al.* (1991) have shown that cultured human keratinocytes produce biologically active NGF. It is thus likely that presumptive pigment cells encounter neurotrophins when they arrive in the developing skin.

Pluripotent neural crest cells and/or their progeny. Our data indicate that SCF alone is trophic for early neural crest cells, that is, for pluripotent and/or their more mature progeny. The combination of SCF and a neurotrophin is trophic for committed melanogenic cells. This is analogous to the hematopoietic system, where SCF acts upon hemopoietic stem cells and on more mature progenitor cells alone and synergistically with other cytokines (reviewed by Broxmeyer *et al.*, 1991). The trophic effect of SCF on neural crest stem cells and/or more mature progenitor cells as observed in the present study is abolished when a neurotrophin is present as well. The underlying mechanism and the developmental potential(s) of the targeted cell type remain to be elucidated. It is conceivable that a change in receptor expression is induced. Since all neurotrophins tested neutralize the SCF effect, p75, the low-affinity NGF receptor that binds all three neurotrophins, may be involved. The low-affinity NGF receptor p75 has been implied in apoptosis (Rabizadeh *et al.*, 1993; Barrett and Bartlett, 1994; Chao, 1995). It is thus conceivable that p75-mediated apoptosis counts for the neutralizing effect of neurotrophins. In this context it is of interest that in the embryo, melanocytes do express p75 (see above). Alternatively, it is important to

TABLE 7
Bromodeoxyuridine Incorporation in the Presence and Absence of SCF and NGF

Type of colony	Experiment No.		Control	SCF	NGF	SCF + NGF
Unpigmented	1	Total	25.6 ± 2.1	58.5 ± 7.1	23.4 ± 2.4	26.7 ± 2.7
		$BrdU^+$	5.7 ± 1.1	10.8 ± 1.6	4.2 ± 0.6	5.7 ± 1.0
		$BrdU^+$/100	28.2 ± 5.7	21.4 ± 3.4	23.0 ± 4.4	21.6 ± 3.7
				($p = 0.31$)	($p = 0.49$)	($p = 0.34$)
	2	Total	28.1 ± 3.4	47.4 ± 4.4	27.9 ± 3.3	24.2 ± 2.8
		$BrdU^+$	3.3 ± 0.9	3.6 ± 0.7	1.5 ± 0.3	2.3 ± 0.5
		$BrdU^+$/100	12.4 ± 2.2	10.3 ± 2.8	7.0 ± 1.4	12.9 ± 3.6
				($p = 0.57$)	($p = 0.04$)	($p = 0.91$)
	3	Total	13.7 ± 1.4	15.4 ± 1.9	14.8 ± 1.2	13.3 ± 1.4
		$BrdU^+$	1.6 ± 0.4	1.0 ± 0.3	1.3 ± 0.4	0.6 ± 0.2
		$BrdU^+$/100	9.4 ± 2.4	7.3 ± 2.4	8.8 ± 3.2	4.8 ± 2.2
				($p = 0.55$)	($p = 0.89$)	($p = 0.16$)
Mixed	1	Total	32.3 ± 4.3	50.6 ± 7.9	31.8 ± 5.4	34.9 ± 5.0
		$BrdU^+$	6.7 ± 1.4	7.5 ± 1.6	4.9 ± 1.2	5.6 ± 1.6
		$BrdU^+$/100	21.2 ± 2.8	19.0 ± 3.2	14.5 ± 2.7	18.0 ± 4.1
				($p = 0.60$)	($p = 0.10$)	($p = 0.51$)
	2	Total	27.0 ± 3.0	52.7 ± 6.8	30.8 ± 5.0	31.0 ± 3.9
		$BrdU^+$	1.9 ± 0.8	2.4 ± 0.6	2.0 ± 0.4	3.1 ± 1.0
		$BrdU^+$/100	7.4 ± 2.1	4.8 ± 1.2	11.0 ± 2.7	10.2 ± 2.9
				($p = 0.30$)	($p = 0.30$)	($p = 0.44$)
	3	Total	17.4 ± 1.5	22.8 ± 2.2	16.8 ± 1.4	15.6 ± 1.2
		$BrdU^+$	1.7 ± 0.5	0.5 ± 0.2	1.3 ± 0.4	0.5 ± 0.2
		$BrdU^+$/100	9.8 ± 2.9	3.1 ± 1.9	7.0 ± 2.0	5.1 ± 2.1
				($p = 0.06$)	($p = 0.42$)	($p = 0.20$)
Pigmented	1	Total	4.6 ± 0.4	4.1 ± 0.3	4.6 ± 0.4	7.8 ± 0.6
		$BrdU^+$	0.6 ± 0.2	0.1 ± 0.1	0.3 ± 0.1	0.7 ± 0.2
		$BrdU^+$/100	14.2 ± 5.1	4.4 ± 2.2	7.4 ± 2.5	8.9 ± 2.7
				($p = 0.09$)	($p = 0.24$)	($p = 0.37$)
	2	Total	6.4 ± 0.7	5.9 ± 0.6	6.3 ± 0.7	11.6 ± 0.8
		$BrdU^+$	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.1
		$BrdU^+$/100	2.2 ± 1.0	2.4 ± 1.4	1.0 ± 0.8	2.4 ± 0.8
				($p = 0.93$)	($p = 0.37$)	($p = 0.90$)
	3	Total	6.1 ± 0.7	7.5 ± 0.7	5.9 ± 0.7	8.6 ± 1.0
		$BrdU^+$	0.3 ± 0.1	0.1 ± 0.04	0.1 ± 0.1	0.3 ± 0.2
		$BrdU^+$/100	7.2 ± 3.8	1.0 ± 0.7	3.3 ± 2.0	6.6 ± 3.8
				($p = 0.12$)	($p = 0.37$)	($p = 0.90$)

Note. On Culture Day 6 (experiments 1 and 2), and on Culture Day 3 (experiment 3) the cells were treated with 10 μM bromodeoxyuridine (BrdU) for 30 min and subsequently stained by indirect immunohistochemistry with anti-BrdU. No statistically significant differences were observed, indicating that SCF is trophic for cells in unpigmented and mixed colonies and the SCF/NGF combination for melanogenic cells, rather than mitogenic. Total, average total number of nuclei per colony; $BrdU^+$, average number of BrdU-positive nuclei per colony; $BrdU^+$/100, average number of BrdU-positive nuclei per 100 nuclei. Cell counts are averages from 25–30 colonies for each colony type in each of the four groups. Statistical analysis is based on two-tailed Student's *t* tests. In order to control for multiple comparisons, *p*-values of $\leqslant 0.02$ are considered indicative of significant differences.

note that quantitative rather than qualitative changes in receptor expression may account for our observations, as the ratio between receptors is important for regulating responsiveness to neurotrophins (Dechant *et al.*, 1995).

Sensory neuron precursors. The number of colonies that contained SSEA-1-immunoreactive sensory neuron precursors was increased in the presence of SCF. Most likely the subset of affected cells is distinct from the one that leads to increased colony size. The fact that both *c-kit* and SCF are expressed in dorsal root ganglia indicates physiological relevance. Carnahan *et al.* (1994) have found that SCF selectively supports survival of small DRG neurons. Most likely the SCF-mediated increase in the number of colonies that contain SSEA-1-immunoreactive cells reflects the trophic action of SCF that has been observed in DRG cultures by Carnahan *et al.* (1994). In the present study, NGF and NT-3, but not BDNF, neutralized the SCF effect on SSEA-1-immunoreactive sensory neuron precursors. The neuro-

TABLE 8
Influence of SCF on Neural Crest Cell Development when Combined with Different Neurotrophins

Type of colony	Experimental conditions							
	Control	SCF	NGF	BDNF	NT-3	SCF + NGF	SCF + BDNF	SCF + NT-3
				Number of colonies per plate ± SEM				
SSEA-1+	2.3 ± 0.5	6.1 ± 0.7 ($p < 0.001$)	1.9 ± 0.7 ($p = 0.64$)	8.6 ± 0.8 ($p < 0.001$)	2.6 ± 0.6 ($p = 0.90$)	2.0 ± 0.3 ($p = 0.62$)	6.4 ± 0.5 ($p < 0.001$)	2.0 ± 0.4 ($p = 0.63$)
Unpigmented	12.3 ± 0.5	11.8 ± 1.0 ($p = 0.64$)	10.1 ± 0.7 ($p = 0.01$)	10.4 ± 1.2 ($p = 0.15$)	9.3 ± 0.7 ($p = 0.002$)	10.0 ± 1.1 ($p = 0.06$)	14.5 ± 1.7 ($p = 0.23$)	11.7 ± 1.0 ($p = 0.59$)
Mixed	8.0 ± 1.0	5.0 ± 0.8 ($p = 0.04$)	6.2 ± 0.8 ($p = 0.19$)	7.5 ± 0.7 ($p = 0.69$)	7.6 ± 0.7 ($p = 0.76$)	7.2 ± 1.1 ($p = 0.61$)	7.0 ± 0.8 ($p = 0.45$)	6.4 ± 1.0 ($p = 0.27$)
Pigmented	33.8 ± 2.6	33.7 ± 2.5 ($p = 0.98$)	35.1 ± 1.9 ($p = 0.69$)	36.3 ± 2.9 ($p = 0.53$)	36.1 ± 2.3 ($p = 0.52$)	33.9 ± 2.4 ($p = 0.98$)	36.1 ± 1.7 ($p = 0.47$)	33.0 ± 1.6 ($p = 0.80$)
Total	54.1 ± 3.2	50.5 ± 3.1 ($p = 0.43$)	51.2 ± 2.4 ($p = 0.48$)	54.2 ± 3.0 ($p = 0.98$)	52.9 ± 2.2 ($p = 0.76$)	51.0 ± 3.2 ($p = 0.50$)	57.6 ± 3.1 ($p = 0.44$)	51.1 ± 1.5 ($p = 0.41$)
				Number of cells per colony ± SEM				
Unpigmented	12.6 ± 1.6	30.3 ± 2.9 ($p < 0.001$)	11.5 ± 1.3 ($p = 0.61$)	17.1 ± 1.9 ($p = 0.07$)	15.2 ± 1.8 ($p = 0.28$)	13.8 ± 1.4 ($p = 0.57$)	13.3 ± 1.4 ($p = 0.71$)	15.0 ± 1.7 ($p = 0.30$)
Mixed	18.9 ± 1.7	46.7 ± 5.2 ($p < 0.001$)	16.9 ± 1.7 ($p = 0.41$)	19.1 ± 1.8 ($p = 0.95$)	17.0 ± 2.1 ($p = 0.47$)	16.7 ± 1.6 ($p = 0.33$)	17.9 ± 1.9 ($p = 0.70$)	15.7 ± 1.4 ($p = 0.14$)
Pigmented	4.4 ± 0.5	4.3 ± 0.5 ($p = 0.96$)	4.4 ± 0.5 ($p = 0.92$)	5.0 ± 0.5 ($p = 0.35$)	4.8 ± 0.6 ($p = 0.57$)	10.2 ± 0.9 ($p < 0.001$)	14.7 ± 1.4 ($p < 0.001$)	9.6 ± 1.0 ($p < 0.001$)

Note. Both control and experimental groups were composed of 10 replicate plates and colony counts are based on counts from 30 colonies for each colony type in each of the eight groups. Statistical analysis is based on Student's two-sided t test. In order to control for multiple comparisons, p-values of $\leqslant 0.009$ are considered as indicative of significant differences. The decrease in the number of unpigmented colonies per plate observed for groups treated with NGF and NT-3 was not reproducible.

trophin-specific response suggests involvement of high-affinity neurotrophin receptors. It also confirms our previous observation that BDNF, but not NGF, is involved in the differentiation of SSEA-1-immunoreactive precursors (Sieber-Blum, 1991). It is conceivable that a TrkB expressing subset of cells is rescued by BDNF from the putative p75-mediated apoptotic response discussed above. However, their increase in number is likely too small to significantly affect colony size. The fact that the promoting effects of SCF and BDNF are neither additive nor synergistic suggests

TABLE 9
Summary of Observations

Factor(s) added	Effect on pluripotent cells and/or their immediate progeny (Number of cells in unpigmented and mixed colonies)	Effect on sensory neuron precursors (Number of unpigmented and mixed colonies that contain SSEA-1-positive cells)	Effect on pigment cells (Number of cells in pigmented colonies)
SCF	Increase	Increase	None
NGF	None	None	None
SCF + NGF	None	None	Increase
SCF →NGF	None	None	Increase
NGF → SCF	None	None	None
BDNF	None	Increase	None
SCF + BDNF	None	Increase	Increase
NT-3	None	None	None
SCF + NT-3	None	None	Increase

Note. (+) Both factors were present throughout the 10 day culture period. (→) The factor listed first was present from Culture Day 0 to Day 5 and then removed, and the second factor was present from Culture Day 5 to Day 10.

that the same subset of sensory neuron precursors is affected by SCF and BDNF. This redundancy possibly explains the lack of deficits in the DRG of mice with mutated SCF or *c-kit* genes.

Melanogenesis. Our data indicate that the combination of SCF and a neurotrophin (NGF, BDNF, or NT-3) promotes survival of melanogenic cells, but that SCF alone has no detectable effect. Therefore, either both SCF and a neurotrophin are necessary for trophic action or SCF-exposed cells become dependent on neurotrophins for survival. Our data favor the latter possibility. The notion of melanotrophic action by a combination of factors is in apparent disagreement not only with observations *in vivo* in mouse mutants, but also with *in vitro* data derived from cultured mouse and avian neural crest cells, which seem to indicate that SCF alone is melanotrophic. The apparent contradictory data can be reconciled as follows. (1) *In vivo* comparisons between wild-type mice and mice that are mutated in *c-kit* or SCF cannot exclude the involvement of additional factors. (2) The *in vitro* approach has the advantage that experiments can be performed in a defined environment. However, to our knowledge, our present *in vitro* study on the role of SCF in neural crest cell development is the first one that has been performed in the complete absence of serum in the culture medium. Previous studies have been performed in culture media that contained 10% fetal bovine serum (Morrison-Graham *et al.,* 1990; Murphy *et al.,* 1992; Morrison-Graham and Weston, 1993). Serum can contain growth factors, including neurotrophins, and thus most likely explains the discrepancies. One exception is the study by Lahav *et al.,* in which it is claimed that the effect of SCF was studied under defined conditions. However, these authors cultured the neural crest cells for 3 days in 10% fetal bovine serum plus 2% chick embryo extract and changed to a SCF-containing defined culture medium on Day 4. In view of the fact that in neural crest cell cultures pigmentation starts at Day 3 and is completed by Day 4, critical stages in melanogenesis are thus likely to be missed with their experimental paradigm. Therefore, the fact that the cells in the study by Lahav *et al.* (1994) have been exposed to serum can explain their observation of an increase in melanocytes in the presence of SCF alone.

Moreover, Lahav *et al.* (1994) have used the early melanocyte marker, MelEM, as a marker for the pigment cell lineage. Since there was concern that in our approach unpigmented melanogenic cells (melanoblasts) are excluded from evaluation, we repeated our experiments using MelEM. The MelEM epitope is on a melanocyte- and liver-specific subunit of the ubiquitous enzyme glutathione S-transferase (Nataf *et al.,* 1995). Based on the fact that *in vivo* MelEM stained pigment cells as well as nearby unpigmented cells, the authors concluded that the unpigmented MelEM-immunoreactive cells must be melanoblasts. However, there is an alternative possibility that was not pursued by Nataf *et al.* (1993). We have performed double labeling experiments and observed that in early cultures the MelEm epitope colocalizes with the neuronal marker, β-III tubulin. This is a strong indication that MelEM is not a marker unique to committed melanogenic cells. Thus, an additional explanation of the discrepancies between our data and those of Lahav *et al.* is suggested: While Lahav and collaborators considered MelEM-immunoreactive cells committed melanoblasts, all or some of these cells are in reality uncommitted precursors that increased in number as a consequence of exposure to SCF. These may be bipotent or pluripotent neural crest cells and may very well be the same or related precursors that lead in our culture system to an increase in the size of unpigmented and mixed colonies in the presence of SCF. This notion of uncommitted precursor cells in the subectodermal mesenchyme is in agreement with our finding of pluripotent neural crest-derived cells in the developing trunk ectoderm (Richardson and Sieber-Blum, 1993; Sieber-Blum *et al.,* 1993).

The observed colocalization of MelEM and neuron-specific β-III tubulin may offer us important new insights into the mechanisms of neural crest cell type specification. It suggests that progenitor cells can express more than one lineage-specific marker. Interestingly, there is again a correlate in hematopoiesis. Various hematopoietic markers that originally were thought to be lineage-specific have later been found to be expressed by cells in other lineages as well. It is thus thought that some markers that are expressed in a lineage-restricted fashion in committed cells can also be transiently coexpressed by multipotent cells and perhaps briefly in the progeny of these cells following the initiation of commitment. Thus ''lineage promiscuity'' could precede lineage fidelity as a normal course of events. Likely candidates are genes that are among the first to be expressed in a lineage (reviewed by Greaves *et al.,* 1986). This concept is reflected in undifferentiated acute leukemias that can coexpress early hematopoietic, myeloid, B lymphoid, and T lymphoid antigens (see, e.g., Meckenstock *et al.,* 1995). The initial coexpression of an early pigment cell marker and an early neuronal marker and the subsequent down-regulation of the lineage-inappropriate marker in more mature cells, as observed in our present study, is reminiscent of the observations made in hematopoiesis.

Other studies on the role of SCF in melanogenesis also indicated the requirement of a second factor (or factors) that act in tandem with SCF. Murphy *et al.* (1992) reported that the production of melanin was not achieved with SCF alone, but also required the presence of the tumor promotor TPA (12-*O*-tetradecanoylphorbol-13-acetate). Moreover, LeCoin *et al.* (1995) reported that production of SCF in the hyperpigmented mutant Silky Fowl is quantitatively and spatially similar to normal embryos and concluded that an additional unidentified factor (or factors) must be involved in the expansion of melanoblasts in the subectodermal region. The spatial and temporal expression of neurotrophins in the developing skin and our current data suggest that neurotrophins represent the, or one of the, postulated factor(s).

In summary, our *in vitro* data suggest that SCF alone and in combination with neurotrophins has multiple actions at different levels of neural crest cell development.

ACKNOWLEDGMENTS

This work was supported by USPHS Grant HD21423 (M.S.-B.), a Research Grant from the Familial Dysautonomia Foundation (M.S.-B.), and a predoctoral fellowship from the American Heart Association (J.L.S.). We thank Drs. J. Winslow, C. Schmelzer, and L. E. Burton, Genentech, for a gifts of human recombinant BDNF and NT-3; Drs. K. Langley and B. Mendiaz, AMGEN, for a gift of chicken SCF; Dr. Anthony Frankfurter, University of Virginia, for a generous gift of TuJ1 antibody; Drs. J. Dodd and T. Jessell, Columbia University, for providing AC-4; Dr. R. Hoffmann, Medical College of Wisconsin, for his advice on statistical analysis, and C. Snyder and D. Generotzky for their help with the illustrations. The MelEM antibody was obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine (Baltimore, MD) and the Department of Biological Sciences, University of Iowa (Iowa City, IA) under Contract N01-HD-6-2915 from the NICHD.

REFERENCES

Anderson, D. M., Lyman, S. D., Baird, A., Wignall, J. M., Eisenman, J., Rauch, C., March, C. J., Boswell, H. S., Gimpel, S. D., Cosman, D., and Williams, D. E. (1990). Molecular cloning of mast cell growth factor, a hematopoietin that is active in both membrane bound and soluble forms. *Cell* 63, 235–243.

Altus, M. S., Berstein, S. E., Russell, E. S., Carsten, A. L., and Upton, A. C. (1971). Defect extrinsic to stem cells in spleen of Steel anemic mice. *Proc. Soc. Exp. Biol. Med.* 138, 985–988.

Barde, Y.-A., Edgar, D., and Thoenen, H. (1982). Purification of a new neurotrophic factor from mammalian brain. *EMBO J.* 1, 549–553.

Baroffio, A., Dupin, E., and Le Douarin, N. M. (1988). Clone-forming ability and differentiation potential of migratory neural crest cells. *Proc. Natl. Acad. Sci. USA* 85, 5325–5329.

Barrett, G. L., and Bartlett, P. F. (1994). The p75 nerve growth factor receptor mediates survival or death depending on the stage of sensory neuron development. *Proc. Natl. Acad. Sci. USA* 91, 6501–6505.

Bennett, D. (1956). Developmental analysis of a mutation with pleiotropic effects in the mouse. *J. Morphol.* 98, 199–233.

Berkemeier, L. R., Winslow, J. W., Kaplan, D. R., Nikolics, K., Goeddel, D. V., and Rosenthal, A. (1991). Neurotrophin-5: A novel neurotrophic factor which activates Trk and TrkB. *Neuron* 7, 857–866.

Birren, S. J., and Anderson, D. J. (1990). A v-*myc*-immortalized sympathoadrenal progenitor cell line in which neuronal differentiation is initiated by FGF but not NGF. *Neuron* 4, 189–201.

Bronner-Fraser, M., and Fraser, S. E. (1988). Cell lineage analysis reveals multipotency of some avian neural crest cells. *Nature (London)* 335, 161–164.

Broxmeyer, H. E., Maze, R., Miyazawa, K., Carow, C., Hendrie, P. C., Cooper, S., Hangoc, G. Vadhan-Raj, S., and Lu, L. (1991). The kit receptor and its ligand, steel factor, as regulators of hemopoiesis. *Cancer Cells* 3, 480–487.

Carnahan, J. F., Patel, D. R., and Miller, J. A. (1994). Stem cell factor is a neurotrophic factor for neural crest-derived chick sensory neurons. *J. Neurosci.* 14, 1433–1440.

Cattaneo, E., and McKay, R. (1990). Proliferation and differentiation of neuronal stem cells regulated by nerve growth factor. *Nature* 347, 762–765.

Chabot, B., Stephenson, D. A., Chapman, V. M., Besmer, P., and Bernstein, A. (1988). The proto-oncogene *c-kit* encoding a transmembrane tyrosine kinase receptor maps to the mouse W locus. *Nature* 335, 88–89.

Chao, M. V. (1995). Ceramide: A potential second messenger in the nervous system. *Mol. Cell. Neurosci.* 6, 91–96.

Cohen, A. M., and Konigsberg, I. R. (1975). A clonal approach to the problem of neural crest determination. *Dev. Biol.* 46, 262–280.

Copeland, N. G., Gilbert, G. J., Cho, B. C., Donovan, P. C., Jenkins, M. A., Cosman, D., Anderson, D., Lyman, S. D., and Williams, E. D. (1990). Mast cell growth factor maps near the S1 locus and is structurally altered in a number of *Steel* alleles. *Cell* 63, 175–183.

Davies, A. M., Bandtlow, C., Heumann, R., Korsching, S., Rohrer, H., and Thoenen, H. (1987). Timing and site of nerve growth factor synthesis in developing skin in relation to innervation and expression of the receptor. *Nature* 326, 353–358.

Dechant, G., Schroepel, A., and Barde, Y.-A. (1995). Neurotrophin receptors on chick sympathetic neurons during the developmental switch from neurotrophin-3 to nerve growth factor responsiveness. *Soc. Neurosci. Abstr.* 21(2), 1300.

Di Marco, E., Marchisio, P. C., Bondanza, S., Franzi, T., Cancedda, R., and De Luca, M. (1991). Growth-regulated synthesis and secretion of biologically active nerve growth factor by human keratinocytes. *J. Biol. Chem.* 266, 21718–21722.

Dodd, J., and Jessell, T. M. (1986). Cell surface glycoconjugates and carbohydrate-binding proteins: possible recognition signals in sensory neurone development. *J. Exp. Biol.* 124, 225–238.

Duff, R. S., Langtimm, C. J., Richardson, M. K., and Sieber-Blum, M. (1991). *In vitro* clonal analysis of progenitor cell patterns in dorsal root and sympathetic ganglia of the quail embryo. *Dev. Biol.* 147, 451–459.

Dunnett, C. W. (1964). New tables for multiple comparisons with a control. *Biometrics* 20, 482–491.

Ernfors, P., Ibanez, C. F., Ebendahl, T., Olson, L., and Persson, H. (1990). Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: Developmental and topographical expression in the brain. *Proc. Natl. Acad. Sci. USA* 87, 5454–5458.

Fraser, S. E., and Bronner-Fraser, M. (1991). Migrating neural crest cells in the trunk of the avian embryo are multipotent. *Development* 112, 913–920.

Fried, W., Chamberlin, W., Knopse, W. H., Husseini, S., and Trobaugh, F. E. (1973). Studies on the defective hematopoietic microenvironment in S1/S1^{d} mice. *Br. J. Haematol.* 24, 643–650.

Geissler, E. N., McFarland, E. C., and Russell, E. S. (1981). Analysis of the pleiotropism at the dominant *white-spotting* (W) locus of the house mouse: A description of ten new W allelles. *Genetics* 97, 337–361.

Geissler, E. N., Ryan, M. A., and Housman, D. E. (1988). The dominant-white spotting (W) locus of the mouse encodes the c-kit proto-oncogene. *Cell* 55, 185–192.

Gershon, M. D., Chalazonitis, A., and Rothman, T. P. (1993). From neural crest to bowel: Development of the enteric nervous system. *J. Neurobiol.* 24, 199–214.

Greaves, M. F., Chan, L. C., Furley, A. J. W., Watt, S. M., and Molgaard, H. V. (1986). Lineage promiscuity in hemopoietic differentiation and leukemia. *Blood* 67, 1–11.

Hamburger, V., and Hamilton, H. (1951). A series of normal stages in the development of the chick embryo. *J. Morphol.* 88, 49–92.

Heuer, J. G., Fatemie-Nainie, S., Wheeler, E. F., and Bothwell, M. (1990). Structure and developmental expression of the chicken NGF receptor. *Dev. Biol.* 137, 287–304.

Hohn, A., Leibrock, J., Bailey, K., and Barde, Y.-A. (1990). Identification and characterization of a novel member of the nerve growth factor-brain-derived neurotrophic factor family. *Nature* 344, 339–341.

Huang, E., Nocka, K., Beier, D. R., Chu, T., Buck, J., Lahm, H.-W., Leder, P., and Besmer, P. (1990). The hematopoietic growth factor KL is encoded by the *Steel* locus and is the ligand for the *c-kit* factor, the product of the W locus. *Cell* 63, 223–233.

Ito, K., and Sieber-Blum, M. (1991). *In vitro* clonal analysis of quail cardiac neural crest development. *Dev. Biol.* 148, 95–106.

Ito, K., Morita, T., and Sieber-Blum, M. (1993). *In vitro* clonal analysis of mouse neural crest development. *Dev. Biol.* 157, 517–525.

Ito, K., and Sieber-Blum, M. (1993). Pluripotent and developmentally restricted neural crest-derived cells in posterior visceral arches. *Dev. Biol.* 156, 191–200.

Kalcheim, C., Carmeli, C., and Rosenthal, A. (1992). Neurotrophin-3 is a mitogen for cultured neural crest cells. *Proc. Natl. Acad. Sci. USA* 89, 1661–1665.

Katsetos, C. D., Karkavels, G., Frankfurter, A., Vlachos, I. N., Vogeley, K., Schober, R., Wechsler, W., and Urich, H. (1994). The stromal Schwann cell during maturation of peripheral neuroblastomas. *Clin. Neuropathol.* 13, 171–180.

Keshet, E., Lyman, S. D., Williams, E. D., Anderson, D. M., Jenkins, N. A., Copeland, N. G., and Parada, L. F. (1991). Embryonic RNA expression of the *c-kit* receptor and its cognate ligand suggest multiple roles in mouse development. *EMBO. J.* 10, 2425–2435.

Kleinman, H., McGoodwin, E. B., Rennard, S. I., and Martin, G. R. (1979). Preparation of collagen substrates for cell attachment: Effect of collagen concentration and phosphate buffer. *Anal. Biochem.* 94, 308–313.

Lahav, R., LeCoin, L., Ziller, C., Nataf, V., Carnahan, J. F., Martin, F. H., and Le Douarin, N. M. (1994). Effect of the Steel gene product on melanogenesis in avian neural crest cell cultures. *Differentiation* 58, 133–139.

Lecoin, L., Lahav, R., Martin, F. H., Teillet, M.-A., and Le Douaring, N. M. (1995). *Steel* and *c-kit* in the development of avian melanocytes: A study of normally pigmented birds and of the hyperpigmented mutant Silky Fowl. *Dev. Dyn.* 203, 106–118.

Le Douarin, N. M. (1982). ''The Neural Crest.'' Cambridge Univ. Press, London.

Lee, M. K., Tuttle, J. B., Rebhun, L. I., Cleveland, D., and Frankfurter, A. (1990). The expression and posttranslational modification of a neuron-specific β-tubulin isotype during chick embryogenesis. *Cell Motil. Cytoskeleton* 17, 118–132.

Levi-Montalcini, R. (1982). Developmental neurobiology and the natural history of nerve growth factor. *Annu. Rev. Neurosci.* 5, 341–362.

Maisonpierre, P. C., Belluscio, L., Squinto, S., Ip, N. Y., Furth, M. E., Lindsay, R. M., and Yancopoulos, G. (1990). Neurotrophin-3: A neurotrophic factor related to NGF and BDNF. *Science* 247, 1446–1451.

Martin, F. H., Suggs, S., Langley, K. E., Lu, H. S., Ting, J., Okino, C., Morris, F. H., McNiece, I. K., Jacobsen, F. W., Mendiaz, E. A., Birkett, N. C., Smith, K. A., Johnson, M. J., Parker, V., P., Flores, J. C., Patel, A. C., Fisher, E. F., Erjavec, H. O., Wypych, J., Sachdev, R. J., Pope, J. A., Leslie, I., Wen, D., Lin, C. H., Cupples, R. L., and Zsebo, K. M. (1990). Primary structure and functional expression of rat and human stem cell factor DNAs. *Cell* 63, 203–211.

Matsui, T., Zsebo, K. M., and Hogan, B. L. M. (1990). Embryonic expression of a hematopoietic growth factor encoded by the *Sl* locus and the ligand for c-kit. *Nature* 347, 667–669.

Mayer, T. C. (1970). A comparison of pigment cell development in albino, steel, and dominant-spotting mutant mouse embryos. *Dev. Biol.* 23, 297–309.

McCulloch, E. A., Siminovitch, L., and Till, J. E. (1964). Spleen-colony formation in anemic mice of genotype W/W^v. *Science* 144, 844–846.

Meckenstock, G., Heyll, A., Schneider, E. M., Hildebrandt, B., Runde, V., Aul, C., Bartram, C. R., Ludwig, W. D., and Schnedier, W. (1995). Acute leukemia coexpressing myeloid, B- and T-lineage associated markers: Multiparameter analysis of criteria defining lineage commitment and maturational stage in a case of undifferentiated leukemia. *Leukemia* 9, 260–264.

Morrison-Graham, K., West-Johnsrud, L., and Weston, J. A. (1990). Extracellular matrix from normal but not *Steel* mutant mice enhances melanogenesis in cultured mouse neural crest cells. *Dev. Biol.* 139, 299–307.

Morrison-Graham, K., Schatteman, G. C., Bork, T., Bowen-Pope, D. F., and Weston, J. A. (1992). A PDGF receptor mutation in the mouse (Patch) perturbs the development of a non-neuronal subset of neural crest-derived cells. *Development* 115, 133–142.

Morrison-Graham, K., and Weston, J. A. (1993). Transient steel factor dependence by neural crest-derived melanocyte precursors. *Dev. Biol.* 159, 346–352.

Motro, B., Van Der Kooy, D., Rossant, J., Reith, A., and Bernstain, A. (1991). Contiguous patterns of *c-kit* and *Steel* expression: Analysis of mutations at the *W* and *Sl* locus. *Development* 113, 1207–1221.

Murphy, M., Reid, K., Williams, D. E., Lyman, S. D., and Bartlett, P. F. (1992). Steel factor is required for maintenance, but not differentiation, of melanocyte precursors in the neural crest. *Dev. Biol.* 153, 396–401.

Nataf, V., Mercier, P., Ziller, C., and Le Douarin, N. M. (1993). Novel markers of melanocyte differentiation in the avian embryo. *Exp. Cell Res.* 207, 171–182.

Nataf, V., Mercier, P., De Nechaud, B., Guillemot, J. C., Capdevielle, J., Lapointe, F., and Le Douarin, N. M. (1995). Melanoblast/melanocyte early marker (MelEM) is a glutathione S-transferase subunit. *Exp. Cell Res.* 218, 394–400.

Nishikawa, S., Kusakabe, M., Yoshinaga, K., Ogawa, M., Hayashi, S., Kunisada, T., era, T., Sakakura, T., and Nishikawa, S. (1991). *In utero* manipulation of coat color formation by a monoclonal anti-c-kit antibody: Two distinct waves of *c-kit*-dependency during melanocyte development. *EMBO J.* 10, 2111–2118.

Noden, D. (1978). Interactions directing the migration and cytodifferentiation of avian neural crest cells. *In* ''Specificity of Embryological Interactions, Receptors, and Recognition'' (D. R. Garrod, Ed.), pp.-3–49. Chapman & Hall, London.

Orr-Urtreger, A., Avivi, A., Zimmer, Y., Givol, D., Yarden, Y., and Lonai, P. (1991). Developmental expression of *c-kit* a proto-oncogene encoded by the W locus. *Development* 109, 911–923.

Peacocke, M., Yaar, M., Mansur, C. P., Chao, M., and Gilchrest, B. A. (1988). Induction of nerve growth factor receptors on cultured human melanocytes. *Proc. Natl. Acad. Sci. USA* 85, 5282–5286.

Qui, F., Ray, P., Brown, K., Barker, P. E., Jhanwar, S., Ruddle, F. H., and Bessmer, P. (1988). Primary structure of c-kit: Relationship with the CSF-1/PDGF receptor kinase family—oncogenic

activation of c-kit involves deletion of extracellular domain and C-terminus. *EMBO J.* 7, 1003–1011.

Rabizadeh, S., Oh, J., Zhong, L.-T., Yang, J., Bitler, C. M., Butcher, L. L., and Bredesen, D. E. (1993). Induction of apoptosis by the low affinity NGF receptor. *Science* 261, 345–348.

Rachel, R. A., and Sieber-Blum, M. (1989). Glutamate-immunoreactive sensory neurons in quail neural crest cell culture. *Brain Res.* 497, 43–50.

Richardson, M. K., and Sieber-Blum, M. (1993). Pluripotent neural crest cells in the developing skin of the quail embryo. *Dev. Biol.* 157, 348–358.

Rosenthal, A., Goeddel, D. V., Nyugen, T., Lewis, M., Shih, A., Laramee, G. R., Nikolics, K., and Winslow, J. W. (1990). Primary structure and biological activity of a novel human neurotrophic factor. *Neuron* 4, 767–773.

Russell, E. S. (1979). Hereditary anemias of the mouse: A review for geneticists. *Adv. Genet.* 20, 357–459.

Scott, S. (1993). Ontogeny, characterization and trophic dependence of AC4/anti-SSEA-1-positive sensory neurons in the chick. *Dev. Brain Res.* 75, 175–184.

Schecterson, L. C., and Bothwell, M. (1992). Novel roles of neurotrophins are suggested by BDNF and NT-3 mRNA expression in developing neurons. *Neuron* 9, 449–463.

Sieber-Blum, M. (1989a). Commitment of neural crest cells to the sensory neuron lineage. *Science* 243, 1608–1611.

Sieber-Blum, M. (1989b). SSEA-1 is a specific marker for the spinal sensory neuron lineage in the quail embryo and in neural crest cell cultures. *Dev. Biol.* 134, 362–375.

Sieber-Blum, M. (1991). Role of the neurotrophic factors BDNF and NGF in the commitment of pluripotent neural crest cells. *Neuron* 6, 949–955.

Sieber-Blum, M., and Chokshi, H. R. (1985). *In vitro* proliferation and terminal differentiation of quail neural crest cells in a defined culture medium. *Exp. Cell Res.* 158, 267–272.

Sieber-Blum, M., and Cohen, A. M. (1980). Clonal analysis of quail neural crest cells: They are pluripotent and differentiate *in vitro* in the absence of non-crest cells. *Dev. Biol.* 80, 96–106.

Sieber-Blum, M., and Sieber, F. (1984). Heterogeneity among early quail neural crest cells. *Dev. Brain Res.* 14, 241–246.

Sieber-Blum, M., and Sieber, F. (1985). In vitro analysis of quail neural crest cell differentiation. *In* ''Culture in the Neurosciences.'' (J. E. Bottenstein and G. Sato, Eds.), pp. 193–222. Plenum, New York.

Sieber-Blum, M., Ito, K., Richardson, M. K., Langtimm, C. J., and Duff, R. S. (1993). Distribution of pluripotent neural crest cells in the embryo and the role of brain-derived neurotrophic factor in the commitment to the primary sensory neuron lineage. *J. Neurobiol.* 24, 173–184.

Silvers, W. K. (1979). White-spotting, patch, and rump-white; steel, flexed tail, splotch and variant-waddler. *In* ''The Coat Colors of Mice: A Model for Gene Action and Interaction,'' pp. 206–241. Springer-Verlag, New York.

Steel, K. P., Davidson, D. R., and Jackson, I. J. (1992). TRP-2/DT, a new early melanoblast marker, shows steel growth factor (c-kit ligand) is a survival factor. *Development* 115, 1111–1119.

Stemple, D. L., Mahanthappa, N. K., and Anderson, D. J. (1988). Basic FGF induces neuronal differentiation, cell division, and NGF dependence in chromaffin cells: A sequence of events in sympathetic development. *Neuron* 1, 517–525.

Vogel, K. S., Marusich, M. F., and Weston, J. A. (1993). Restriction of neurogenic ability during neural crest cell differentiation. *J. Neurobiol.* 24, 162–171.

Weston, J. A. (1970). The migration and differentiation of neural crest cells. *Adv. Morphog.* 8, 41–114.

Weston, J. A. (1991). Sequential segregation and fate of developmentally restricted intermediate cell populations in the neural crest lineage. *Curr. Top. Dev. Biol.* 25, 133–153.

Williams, D. E., Eisenman, J., Baird, A., Rauch, C., van Ness, K., March, C. J., Park, L. S., Martin, U., Mochizuki, D. Y., Boswell, H. S., Burgess, G., Cosman, D., and Lyman, S. D. (1990). Identification of a ligand for the *c-kit* proto-oncogene. *Cell* 63, 167–174.

Yaar, M., Grossman, K., Eller, M., and Gilchrest, B. A. (1991). Evidence for nerve growth factor-mediated paracrine effects in human epidermis. *J. Cell Biol.* 115, 821–828.

Yaar, M., Eller, M. S., DiBenedetto, P., Reenstra, W. R., Zhai, S., McQuaid, T., Archambault, M., and Gilchrest, B. (1994). The trk family of receptors mediates nerve growth factor and neurotrophin-3 effects in melanocytes. *J. Clin. Invest.* 94, 1550–1562.

Yamada, K. M. (1982). Isolation of fibronectin from plasma and cells. *In* ''Immunochemistry of the Extracellular Matrix.'' (H. Furthmayr, Ed.), Vol. 1, pp. 111–123. CRC Press, Boca Raton, FL.

Zhang, J.-M., Winslow, J. W., and Sieber-Blum, M. (1993). Role of neurotrophin-3 (NT-3) in the expression of the adrenergic phenotype by neural crest cells. *Soc. Neurosci. Abstr.* 19, 863.

Zhang, J. M., and Sieber-Blum, M. (1992). Anti-proliferative and neurogenic effects by TGFB1 on neural crest cells override mitogenic and adrenogenic effects exerted by the bFGF/NGF peptide factor combination. *Mol. Biol. Cell* 3, 285a.

Zsebo, K., Wypych, J., McNiece, I. K., Lu, H. S., Smith, K. A., Karkare, S. B., Sachdev, R. K., Yuschenkoff, V. N., Birkett, N. C., Williams, L. R., Satyagal, V. N., Tung, W., Bosselman, R. A., Mendiaz, E. A., and Langley, K. L. (1990a). Identification, purification, and biological characterization of hematopoietic stem cell factor from buffalo rat liver-conditioned medium. *Cell* 63, 195–201.

Zsebo, K. M., Williams, D. A., Geissler, E. N., Broudy, V. C., Martin, F. H., Atkins, H. L., Hsu, R. Y., Birkett, N. C., Okine, K. H., Murdock, D. C., Jacobsen, R. W., Langley, K. E., Smith, K. A., Takeishi, T., Cattanach, B. M., Galli, S. J., and Suggs, S. V. (1990b). Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. *Cell* 63, 213–224.

Received for publication January 6, 1995
Accepted December 2, 1995